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Enrichment of ILC Populations in Gingival Tissue

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A supplemental appendix to this article is available online.

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### Enrichment of Innate Lymphoid Cell Populations in Gingival Tissue

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#### Abstract

Innate lymphoid cells (ILCs) are a population of lymphocytes that act as the first line of immunologic defense at mucosal surfaces. The ILC family in the skin, lungs, and gastrointestinal tissues has been investigated, and there are reports of individual subsets of ILCs in the oral tissues. We sought to investigate the whole ILC population (group 1, 2, and 3 subsets) in the murine gingivae and the lymph nodes draining the oral cavity. We show that ILCs made up a greater proportion of the whole CD45+ lymphocyte population in the murine gingivae ( $0.356\% \pm 0.039\%$ ) as compared with the proportion of ILCs in the draining lymph nodes ( $0.158\% \pm 0.005\%$ ). Cytokine profiling of the ILC populations demonstrated different proportions of ILC subsets in the murine gingivae versus the regional lymph nodes. The majority of ILCs in the draining lymph nodes expressed IL-5, whereas there were equal proportions of IFN- $\gamma$ - and IL-5 expressing ILCs in the oral mucosa. The percentage of IL-17+ ILCs was comparable between the murine gingivae and the oral draining lymph nodes. These data suggest an enrichment of ILCs in the murine gingivae, and these ILCs reflect a cytokine profile discrepant to that of the local draining lymph nodes. These studies indicate diversity and enrichment of ILCs at the oral mucosal surface. The function of ILCs in the oral cavity remains to be determined; here, we provide a premise of ILC populations that merits future consideration in investigations of mouse models and human tissues.

#### Keywords

oral mucosa, gingivae, oral draining lymph nodes, cytokines

## Introduction

The balance between the host immune response and the external environment is essential in maintaining a healthy state. Inappropriate barrier immune responses at the oral mucosa may drive the onset and progression of oral inflammatory diseases such as gingivitis and periodontitis ([Lamont and Hajishengallis 2015](#)).

In humans, myriad mechanisms form a first line of innate immunity to microbial challenges at the oral mucosal barrier, including migrating and resident granulocytes/neutrophils, a network of antigen-presenting cells, and a small family of innate lymphoid cells (ILCs; [Dutzan et al. 2016](#)). ILCs constitute a recently identified family of lymphocytes that lack characteristic lineage markers. ILCs are found in secondary lymphoid tissues and at mucosal surfaces of the lung and gastrointestinal (GI) tract ([Sonnenberg and Artis 2015](#)). ILCs have emerged as important regulators in tissue homeostasis and mucosal barrier control of commensal and pathogenic microorganisms ([Sonnenberg et al. 2012](#); [Hepworth et al. 2015](#); [Klose and Artis 2016](#)). The ILC family encompasses 3 functional groups: group 1, ILC1s and natural killer cells; group 2, ILC2s; and group 3, ILC3s ([McKenzie et al. 2014](#)). Human and mice ILC subsets have been defined by their cell surface markers and transcription factor expression; it is widely understood that all ILCs are lineage negative (Lin<sup>−</sup>) and express the interleukin 7 $\alpha$  (IL-7 $\alpha$ ) receptor (CD127), while “conventional” natural killer cells, although Lin<sup>−</sup>, do not express CD127 ([Eberl et al. 2015](#)).

CD117 (c-KIT), the mast/stem cell growth factor receptor, and NKp46 (CD355), the natural cytotoxicity triggering receptor 1 (NCR1), have been identified as markers for phenotyping ILCs into subsets. ILCs are heterogeneous, and the expression of CD117 and NKp46 varies depending on anatomic location ([Spits et al. 2013](#); [Ealey and Koyasu 2017](#); [Simoni and Newell 2017](#)).

The ILC subsets possess unique cytokine profiles that parallel those of T-helper cells: group 1 ILCs (ILC1) release interferon  $\gamma$  (IFN- $\gamma$ ); group 2 ILCs (ILC2) secrete group 2 cytokines, such as IL-4 and IL-5; and group 3 ILCs (ILC3) generate IL-17 and/or IL-22 ([Spits et al. 2013](#)). Group 1 ILCs promote immunity to intracellular bacteria and parasites ([Klose et al. 2014](#); [Klose and Artis 2016](#)). ILC2s facilitate antihelminth and antiviral immunity ([Chang et al. 2011](#); [Moro et al. 2010](#); [Neill et al. 2010](#)) and are involved in orchestrating the immune response to allergens ([Drake and Kita 2014](#); [Halim et al. 2014](#)). ILC3s augment the removal of extracellular bacteria and fungi ([Cella et al. 2009](#); [Killig et al. 2014](#); [Van Maele et al. 2014](#)).

The importance of ILCs as the first line of defense at barrier sites has been highlighted in different murine models of mucosal infection. During infection with the gram-positive respiratory pathogen *Streptococcus pneumoniae*, lung-resident ILC3s produced elevated levels of IL-17 and IL-22—2 cytokines essential in mucosal defense ([Van Maele et al. 2014](#)). Similarly, IL-17 and IL-22-production by ILC3s was observed during gut infections with *Citrobacter rodentium*, a gram-negative enteric bacterium that causes experimental colitis ([Sonnenberg et al. 2012](#); [Rankin et al. 2016](#)). Furthermore, loss of IL-5-producing lung-resident ILC2s in a murine model of influenza A acute viral infection resulted in exacerbated disease phenotype ([Monticelli et al. 2011](#)). In the Peyer's patches of the gut, in vivo depletion of ILC1s and ILC3s interfered with the anatomic containment of commensal bacteria, indicating an essential role for ILCs in bacterial homeostasis in the gut ([Hashiguchi et al. 2015](#)). Other studies described an essential role for IFN- $\gamma$ -producing ILC1s in *Campylobacter jejuni*-induced colitis ([Muraoka et al. 2016](#)).

Over recent years, the ILC family has been the subject of extensive research of the skin, lungs, and GI tract, but the role of ILCs in the oral mucosa is not fully understood. To our knowledge,

ILCs (belonging largely to the ILC1 subset) have been identified in human gingival tissue ([Dutzan et al. 2016](#)), but a description of ILC populations in murine gingivae is restricted to the IL-17-producing ILC3 subset ([Moutsopoulos et al. 2014](#); [Pandiyani et al. 2014](#)). Here, we sought to comprehensively characterize the ILC compartment within the murine gingivae and cervical oral draining lymph nodes (dLNs).

## Methods

### Animals

Female BALB/c and C57BL/6 mice (both Envigo) were maintained in specific pathogen-free conditions with ad libitum access to food and water at the University of Glasgow. All animal work was approved by local research ethics committees and performed under license in accordance with UK Home Office regulations and conforms to the ARRIVE guidelines.

### Tissue Processing

Preparation of gingival tissue single-cell suspensions followed a similar protocol as previously described ([Mizraji et al. 2013](#)). Single-cell suspensions were pooled from 4 or 5 mice per experiment for immediate phenotyping of ILCs or 9 to 11 mice per experiment for cytokine profiling of ILCs.

Single-cell suspensions were obtained from 4 superficial cervical oral dLNs from each mouse by mechanically mashing the tissue through 40- $\mu$ m cell strainers into sterile cRPMI (RPMI-1640 with 10% fetal calf serum, 1% penicillin streptomycin, 1% l-glutamine; Gibco).

### Flow Cytometry

For immediate phenotyping of ILCs, a 1/200 dilution of Fc block (anti-mouse CD16/32; eBioscience) in phosphate-buffered saline was added; then, cells were stained with fluorochrome-labeled cell surface antibodies against CD3, B220, CD11b, CD11c, Ly-6G (all Alexa Fluor 488; lineage “dumpgate”), CD127-phycoerythrin (PE), NKp46-allophycocyanin (APC), viability dye eFluor450 (all eBioscience), CD45–brilliant violet (BV510), and CD117-APC-Cy7 (Biolegend). For cytoplasmic intracellular cytokine staining of the ILCs, cells were stimulated in cRPMI media supplemented with 30 ng/mL of phorbol myristate acetate, 500 ng/mL of ionomycin (both Sigma Aldrich), and GolgiPlug as specified by the intracellular cytokine staining protocol of BD Biosciences. Following stimulation, cells were stained with antibodies specific to CD3, B220, CD11b, CD11c, Ly-6G, and CD45 and with a viability dye, as described earlier, in addition to CD127-PE or CD127-APC (eBioscience). Cells were then fixed and permeabilized with the intracellular fixation and permeabilization buffer set (eBioscience) and stained with fluorochrome-labeled antibodies specific for IFN- $\gamma$  and IL-17 (both PE-Cy7; eBioscience) or IL-5-PE (eBioscience). Positive and negative gates were applied with reference to “fluorescence minus one” or unstimulated controls. Cells were analyzed with a MACS Quant (Miltenyi Biotec) flow cytometer and the data analyzed with FlowJo (Tree Star Inc.).

### Statistical Analyses

Normally distributed data were analyzed by 2-tailed Student's *t* test to compare the means of 2 samples or multiple-comparison 1-way analysis of variance to compare the means of >2 samples with GraphPad Prism 7. Values are presented as mean  $\pm$  SEM.

## Results

Single-cell suspensions from the gingivae and oral dLNs were isolated and stained for CD3, B220, CD11b, CD11c, Ly-6G, CD45, CD127, CD117, and NKp46. Dead cells were excluded with a Live/Dead Viability dye ILCs were initially identified by positive expression of CD45, a marker present on all hematopoietic cells (including ILCs) and then further characterized per a lack of expression of common lineage markers (Lin<sup>-</sup>; CD3, B220, CD11b, CD11c, Ly-6G) in conjunction with expression of CD127 (IL-7 receptor  $\alpha$ ) as seen in Figure 1A (full gating strategy shown in Appendix Fig. 1). The proportion of total ILCs of the whole CD45<sup>+</sup> lymphocyte population was compared between gingivae and dLNs. There was a significantly higher percentage of total ILCs in the gingivae as compared with the oral dLNs (Fig. 1B; 0.356%  $\pm$  0.039% total ILCs of the CD45<sup>+</sup> lymphocyte population for gingivae vs. 0.158%  $\pm$  0.005% for oral dLNs,  $P < 0.001$ ).

Figure 1.

Percentage of innate lymphoid cells (ILCs) from CD45<sup>+</sup> lymphocytes in cervical draining lymph nodes (dLNs) and gingivae. Superficial cervical oral dLNs and gingivae were harvested from BALB/c mice and processed, with single-cell suspensions stained for antibodies prior to analysis by flow cytometry. (A) ILCs were identified as CD45<sup>+</sup>, lineage<sup>-</sup> (Lin<sup>-</sup>; CD3<sup>-</sup>, B220<sup>-</sup>, CD11b<sup>-</sup>, CD11c<sup>-</sup>, Ly-6G<sup>-</sup>), and CD127<sup>+</sup> lymphocytes. For full gating strategy, see Appendix Figure 1. Flow cytometry plots are from a representative gingivae cell sample. (B) Percentage of ILCs from the whole CD45<sup>+</sup> lymphocyte population in the oral dLNs (clear bars) versus gingivae (shaded bars). Data are presented as mean percentage  $\pm$  SEM of CD45<sup>+</sup> lymphocyte population, representing a 6 independent experiments ( $n$  = oral dLNs, 4 or 5 mice per group/experiment, gingivae pooled from a 4 or 5 mice per group/experiment). Statistical significance determined by 2-tailed unpaired  $t$  test (\*\* $P < 0.001$ ). FSC-A, forward scatter area.

CD45<sup>+</sup>, Lin<sup>-</sup>, CD127<sup>+</sup> cells were further characterized by expression of CD117 (c-KIT; i.e., the mast/stem cell growth factor receptor) and NKp46 (CD355; i.e., NCR1) (Appendix Fig. 1; summarized in Fig. 2A). Identification of ILCs into possible “subsets” with CD117 and NKp46 is described in Appendix Table 1. There was no significant difference in the percentages of CD117<sup>-</sup>, NKp46<sup>-</sup> ILCs in the gingivae as compared with the oral dLNs (Appendix Fig. 2A; 0.182%  $\pm$  0.038% vs. 0.128%  $\pm$  0.010%,  $P < 0.194$ ). However, there was a significantly higher percentage of CD117<sup>+</sup>, NKp46<sup>-</sup> ILCs (Appendix Fig. 2B; 0.1346%  $\pm$  0.0187% vs. 0.0077%  $\pm$  0.0005%,  $P < 0.0001$ ), CD117<sup>-</sup>, NKp46<sup>+</sup> ILCs (Appendix Fig. 2C; 0.0513%  $\pm$  0.0039% vs. 0.0169%  $\pm$  0.0019%,  $P < 0.0001$ ), and CD117<sup>+</sup>, NKp46<sup>+</sup> ILCs (Appendix Fig. 2D; 0.0208%  $\pm$  0.0036% vs. 0.0060%  $\pm$  0.0007%,  $P < 0.01$ ) in the gingivae versus the oral dLNs. Collectively, these data suggest a proportional enrichment of ILCs at the gingival mucosal surface as compared with the oral dLNs.

To determine the relationship between the CD117 $\pm$ , NKp46 $\pm$  ILC populations (identified with plots in Fig. 2A, full gating strategy in Appendix Fig. 1) in the gingivae and oral dLNs, the proportion of these ILCs from the whole ILC population (e.g., CD45<sup>+</sup>, Lin<sup>-</sup>, CD127<sup>+</sup> lymphocytes) was also compared. The ILC subsets (e.g., CD117 $\pm$ , NKp46 $\pm$  ILCs) of the whole ILC population were more diverse in the gingivae than in the oral dLNs. The majority of ILCs in the gingivae and lymph nodes were CD117<sup>-</sup>, NKp46<sup>-</sup> ILCs (Fig. 2B; 45.57%  $\pm$  4.40% vs. 79.99%  $\pm$  0.95%, respectively,  $P < 0.0001$ ), while the percentage of CD117<sup>+</sup>, NKp46<sup>-</sup> ILCs was significantly higher in the gingivae than the oral dLNs (Fig. 2C; 35.19%  $\pm$  3.84% vs. 5.03%  $\pm$  0.69%,  $P < 0.0001$ ). There was no statistically significant difference between the percentage of CD117<sup>-</sup>, NKp46<sup>+</sup> ILCs in either tissue (Fig. 2D; 13.81%  $\pm$  1.70% [gingivae] vs. 10.53%  $\pm$  0.59% [lymph node],  $P = 0.0978$ ) nor CD117<sup>+</sup>, NKp46<sup>+</sup> ILCs in the gingivae and oral dLNs (Fig. 2E; 5.43%  $\pm$  0.79% vs. 3.93%  $\pm$  0.59%, respectively,  $P = 0.1593$ ). These data suggest that the majority of ILCs in the oral cavity are CD117<sup>-</sup> and NKp46<sup>-</sup> (Fig. 3F, G,

blue segment; ~45.57% in the gingivae and ~79.99% in the oral dLNs), although a significant proportion of ILCs in the gingivae is phenotypically CD117+ and NKp46– (Fig. 3G, red segment; ~35.19%).

Figure 2.

Proportion of subsets of innate lymphoid cells (ILCs) from the CD45+, lineage– (Lin–), CD127+ population in murine cervical draining lymph nodes (dLNs) and gingivae. Superficial cervical oral dLNs and gingivae were harvested from BALB/c mice and single-cell suspensions analyzed by flow cytometry. (A) Four populations of ILCs (CD45+, Lin–, CD127+ lymphocytes) were identified with CD117 and NKp46. Flow cytometry plots are from a representative gingivae cell sample. The percentage of each ILC subset as a proportion of the ILC population, compared between the dLNs (clear bars) and gingivae (shaded bars): (B) CD117–, NKp46–; (C) CD117+, NKp46–; (D) CD117–, NKp46+; (E) CD117+, NKp46+. The diversity of the ILC populations: (F) dLNs and (G) gingivae. (B–E) The mean percentage  $\pm$  SEM of CD45+, Lin–, and CD127+ lymphocyte population, representing 6 independent experiments ( $n$  = oral dLNs, 4 or 5 mice per group/experiment, gingivae pooled from 4 or 5 mice per group/experiment). Statistical significance determined by 2-tailed unpaired  $t$  test (\*\*\*\* $P$  < 0.0001).

We next characterized the cytokine profiles of ILCs via intracellular cytokine staining (full gating strategy shown in Appendix Fig. 3). Figure 3A shows the final flow cytometry plots used for identification of IFN- $\gamma$ -producing ILCs (group 1 ILCs), IL-5-producing ILCs (ILC2s), and IL-17-producing ILCs (ILC3s; summarized in Appendix Table 1). We also investigated cytokine expression in the total CD45+ population and compared this with the cytokine expression by the ILCs. The majority of the CD45+ lymphocytes in the oral dLNs expressed IFN- $\gamma$  (Fig. 3B, blue segment; 79.26%  $\pm$  4.45%), with a significantly lower proportion of IL-5-producing cells (Fig. 3B, red segment; 11.45%  $\pm$  3.36%,  $P$  < 0.0001) and IL-17+ cells (Fig. 3B, green segment; 9.29%  $\pm$  1.41%,  $P$  < 0.0001). The cytokine profile of the total CD45+ lymphocytes in the gingivae was similar to that of the oral dLNs, with the majority of cells expressing IFN- $\gamma$  (Fig. 3C, blue segment; 77.69%  $\pm$  1.50%). Compared with the IFN- $\gamma$  producing cells, there were fewer IL-5+ cells (Fig. 3C, red segment; 3.65%  $\pm$  0.40%,  $P$  < 0.0001) and more IL-17-producing cells (Fig. 3C, green segment; 18.66%  $\pm$  1.9%,  $P$  < 0.0001). The ILC subsets in gingivae and dLNs showed a quite different distribution when compared with the CD45+ population. There was a significantly greater proportion of IL-5-producing ILCs (Fig. 3D, red segment; 62.55%  $\pm$  1.48%) isolated from the oral dLNs than IFN- $\gamma$ + ILCs (Fig. 3D, blue segment; 19.36%  $\pm$  1.35%,  $P$  < 0.0001) or IL-17 producing ILCs (Fig. 3D, green segment; 18.09%  $\pm$  2.26%,  $P$  < 0.0001), suggesting that the ILC2 subset was the most prominent ILC cell type in the dLN. There was an equal proportion of IFN- $\gamma$ - and IL-17-producing ILCs in the dLNs ( $P$  = 0.9969). In the gingivae, there was an equal proportion of IFN- $\gamma$ + ILCs (Fig. 3E, blue segment; 42.27%  $\pm$  6.63%) and IL-5+ ILCs (Fig. 3E, red segment; 39.60%  $\pm$  4.12%) and a significantly lower proportion of IL-17-producing ILCs (Fig. 3E, green segment; 18.13%  $\pm$  2.50%,  $P$  < 0.001). Unlike the cytokine profile of the CD45+ lymphocytes, the proportion of IFN- $\gamma$ +, IL-5+, and IL-17+ ILCs differed between the ILCs resident in the 2 oral tissues. The cytokine production of the total lymphocyte population (all CD45+) was similar in the gingivae and the dLN (Fig. 3F). However, there were marked differences in the cytokine expression of the ILC population in the gingivae versus the dLN. There was a significantly greater proportion of IFN- $\gamma$ -producing ILCs in the gingivae than the oral dLNs (Fig. 3G, 42.27%  $\pm$  6.63% vs. 19.36%  $\pm$  1.35%, respectively,  $P$  < 0.001). The population of IL-5+ ILCs was significantly reduced in the gingivae than the oral dLNs (Fig. 3G; 39.60%  $\pm$  4.12% vs 62.55%  $\pm$  1.48%, respectively,  $P$  < 0.001). The proportion of IL-17+ ILCs was comparable in gingivae and oral dLNs (Fig. 3G; 18.13%  $\pm$  2.50% vs. 18.09%  $\pm$  2.26%, respectively  $P$  = 0.9999). Appendix Table 2 presents a summary of the statistical significance between ILC subsets in the gingivae and oral dLNs. Taken

together, these data suggest that the gingivae-resident ILCs have a more diverse cytokine profile than the population of ILCs in the oral dLNs and that the cytokine profile of the ILCs is distinct to that of the total CD45+ population in both tissues.

### Figure 3.

Comparison of the proportion of cytokine-producing innate lymphoid cells (ILCs) in the murine gingivae and oral dLNs. Superficial cervical oral draining lymph nodes (dLNs) and gingivae were harvested from C57BL/6 mice and processed to obtain single-cell suspensions; then, cells were stimulated with PMA/ionomycin and protein release inhibitors for 6 h prior to staining for innate lymphoid cell (ILC) markers and cytokines. **(A)** ILCs (CD45+, lineage- [Lin-], CD127+ lymphocytes) were divided into 3 subsets according to production of IFN- $\gamma$  (ILC1s), IL-5 (ILC2s), and IL-17 (ILC3s). Plots show gingival cells from a representative experiment. **(B–E)** Mean percentage of each cytokine-producing cell type from the cytokine-producing population in both oral tissues: CD45+ lymphocytes and ILCs in dLNs and gingivae. The mean proportion  $\pm$  SEM of cytokine-producing (IFN- $\gamma$ , IL-5, and IL-17) **(F)** CD45+ lymphocytes and **(G)** ILCs. Data represent 2 independent experiments (dLNs pooled from 4 or 5 mice per group/experiment) and gingivae (gingivae pooled from 9 to 11 mice per group/experiment). **(F, G)** Statistical significance determined by multiple-comparison 1-way analysis of variance ( $***P < 0.001$ ,  $****P < 0.0001$ ). FSC-A, forward scatter area.

### Discussion

We demonstrate here that the ILC compartment is enriched and more diverse and has a different cytokine profile at the gingival mucosal surface as compared with that of the oral dLNs. The majority of the ILCs in the oral dLNs and gingivae were CD117-, NKp46-, although there was a substantial population of CD117+, NKp46- ILCs at the mucosal surface. This cell surface phenotype suggested that oral-resident ILCs were mainly ILC1s. Upon further investigation with cytokine analysis, the ILCs in the oral dLNs largely produced IL-5, indicating that these ILCs were predominantly part of the type 2 subset, with an equal distribution of IFN- $\gamma$ - and IL-17-producing ILCs. In the gingivae, there was a relatively similar proportion of IFN- $\gamma$ + and IL-5+ ILCs. Such a discrepancy in cell surface marker and cytokine analysis in oral-resident ILCs may have arisen from 1) differences in staining methods for ex vivo versus in vitro stimulated cells—that is, the cytokine production was analyzed after a period of in vitro stimulation, whereas the cell surface phenotype was analyzed immediately ex vivo—and/or 2) the heterogenous nature and potentially plasticity of ILCs ([Simoni and Newell 2017](#)). Ideally, large numbers of purified ILCs would be obtained from oral tissues and studied over time with different stimulation conditions. Currently, there are no methods of growing these cells in vitro, and their isolation into “pure” populations for in vitro study is extremely challenging given the small numbers and proportions of cells in tissue. Nonetheless, we show that the cytokine profile of the ILCs differed from the whole CD45+ lymphocyte population in the oral dLNs and gingivae; IFN- $\gamma$  was evidently the main cytokine produced by the CD45+ lymphocytes in both tissues.

The ILC compartment is enriched at mucosal surfaces, such as the respiratory and GI tracts and the skin, as compared with regional draining lymph nodes ([Sonnenberg and Artis 2015](#); [Kim et al. 2016](#)) (percentage of ILCs of total CD45+ lymphocytes: 3.7% for lung, 2.5% for small intestine lamina propria, 1.8% for large intestine lamina propria, and 3.5% for skin vs. <0.2% for regional draining lymph nodes). However, there is no work describing an enrichment of ILCs at the oral mucosa as discussed here. As previously shown, ILC populations are more diverse at the mucosal surface of the lower GI tract (e.g., the lamina propria and intraepithelial compartment of the small and large

intestines) as compared with the draining mesenteric lymph nodes ([Kim et al. 2016](#)). The data presented here suggest that a similar phenomenon occurs in the oral mucosa, with an enrichment of ILCs and a more diverse ILC population in the gingivae as compared with the oral dLNs. It is postulated that the increased number of ILCs at mucosal surfaces reflects the importance of these cells as a first-line regulation of innate and adaptive immunity to commensal and harmful microbiota, specifically in the GI tract and lungs ([Klose and Artis 2016](#)). Immune tolerance in CD4+ T cells to commensal bacteria is essential in maintaining tissue homeostasis in the GI tract ([Belkaid et al. 2013](#)), a mechanism controlled in part by communication with gut-resident ILCs (Hepworth et al. 2013). It may be that a similar cross-talk between CD4+ T cells and ILCs is present in the oral cavity, given the vast population of microbes inhabiting the environment, including many commensal bacteria and fungi. Thus, the enrichment and enhanced diversity of the ILC populations in the gingivae may be expected given the constant microbial perturbations to the oral mucosal surface.

It is generally accepted that the majority of ILCs are tissue-resident lymphocytes incapable of circulating around the body. Parabiotic studies with 2 conjoined adult mice that share 1 blood circulatory system demonstrated that ILCs in the small intestine lamina propria, salivary gland, lung, and adipose tissue are strictly tissue-resident cells and do not pass from one mouse to the other ([Gaisteiger et al. 2015](#); [O'Sullivan et al. 2016](#)). Tissue-resident ILCs will expand locally under physiologic conditions in response to immunologic perturbations at mucosal surfaces and are only replenished in small numbers by hematogenous precursors and/or circulating mature ILCs ([Gaisteiger et al. 2015](#)). Nonetheless, other evidence suggests that a small population of ILCs is migratory and can traffic from the mucosal surface to secondary lymphoid organs through the expression of chemokine and homing receptors ([Mackley et al. 2015](#); [Kim et al. 2016](#)). In the gingivae, other lymphocytes (e.g., T cells) can migrate to the secondary lymphoid tissue ([Yamazaki et al. 2012](#)). Currently, it is not known whether the gingivae-resident ILCs are capable of migrating to the oral dLNs and, if so, which subsets would undergo this migration and under what circumstances. Future work will ideally allude to such phenomena, which may also begin

For the purposes of these investigations, we characterized ILCs into 3 “conventional” subsets in accordance with the production of cytokines: IFN- $\gamma$  (ILC1s), IL-5 (ILC2s), and IL-17 (ILC3s). [Dutzan et al. \(2016\)](#) proposed that the most abundant ILC subtype in the human gingivae was strictly IFN- $\gamma$ -producing ILC1s, with only a small percentage of IL-17+ ILC3s. They did not detect any ILC2 populations in the human gingivae. Here we show that in the murine gingivae, there was a relatively equal proportion of ILC1s and ILC2s—based on their generation of IFN- $\gamma$  and IL-5, respectively—and a further population of IL-17+ ILC3s. The variation in the gingivae-resident ILC subsets between mice and human may represent a true species-specific difference in ILC population distribution or an environmental consequence of variable microbial challenge between humans and specific pathogen-free mice. This discrepancy may be evidence of further anomalies in the innate immune response in mice versus humans, with implications for the interpretation of animal model data ([Zschaler et al. 2014](#)). Interestingly, previous studies that identified ILCs in the murine oral mucosa focused on the ILC3 population, with no work, to our knowledge, describing the ILC1 or ILC2 subsets within the tissue. Gingivae-resident ILC3s were identified as a source of IL-17 in an experimental model of leukocyte adhesion deficiency type I periodontitis ([Moutsopolous et al. 2014](#)), and small populations of ILC3s were found in oral mucosal lymphocytes isolated from the tongue, gingival, and palatal tissues ([Pandiyan et al. 2014](#)). To the best of our knowledge, this is the first appraisal of the whole ILC compartment within the murine gingival tissue, including the identification of ILC1s and ILC2s.

The identification of IFN- $\gamma$ -, IL-5-, and IL-17-producing ILCs in the gingival tissue may further complicate regulation of homeostasis and pathways involved in inflammation and infection at the oral mucosa. Nonetheless, the presence of such cells in the oral mucosa suggests that they may play a



role in oral health and diseases. Future work may merit the consideration of comparing ILC populations within other oral mucosal surfaces, such as the tonsils, tongue, and/or palatal tissues, to further aid future investigations in oral immunology. Ultimately, understanding the function of the complex network of different lymphocytes resident in the oral cavity will aid future work in developing novel therapeutics to target the myriad of oral diseases associated with microbial dysbiosis, infection, or inflammation.

#### Author Contributions

J.L. Brown, J. Butcher, S. Culshaw, contributed to conception, design, data acquisition, analysis and interpretation, drafted and critically revised the manuscript; L. Campbell, J. Malcolm, A.A. Planell, Contributed to data acquisition and interpretation, and critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

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